THE PHYSIOLOGY OF INSECT DIAPAUSE. VI. EFFECTS OF TEMPERATURE, OXYGEN TENSION, AND METABOLIC INHIBITORS ON *IN VITRO* SPERMATOGENESIS IN THE CECROPIA SILKWORM [1]

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Throughout the previous papers of this series of studies of the Cecropia silkworm it has been necessary to infer the metabolic reactions of the insect's tissues from the effects of various agents and experimental conditions on the whole or subdivided organism. Manifestly, it would be highly desirable to test these findings on an isolated tissue. Fortunately, this objective is now accessible by virtue of the successful *in vitro* culture of the male sex cells, as demonstrated by Schmidt and Williams (1953). As these investigators have shown, the pupal spermatocytes of the insect, when cultured in simple hanging-drop preparations, undergo meiosis followed by a remarkable differentiation into spermatids and spermatozoa.

In the present investigation we have attempted to define the effects of temperature, oxygen tension, and certain metabolic inhibitors on *in vitro* spermatogenesis. Agents were selected for study whose actions on intermediary metabolism had previously been defined.

Materials and Methods

1. Culture methods

The methods utilized in culturing the spermatocytes were modifications of those described by Schmidt and Williams (1953). In order to block the tyrosine-tyrosinase reaction, several crystals of phenylthiourea (twice recrystallized from the Eastman product) were added to each sample of blood. Pairs of testes were removed from either diapausing or brainless diapausing male pupae and placed in a depression slide containing several drops of hormone-containing blood from a Cecropia pupa that had just initiated adult development. Each testis was then torn open, releasing thousands of spermatocytal cysts into the blood. The resulting suspension was transferred to a sterile test tube and additional active blood added to a volume of two ml. Each such suspension contained about 100 to 300 cysts per drop. The tubes were then plugged with cotton and stored at 2° C. until used. While failing to develop at this low temperature, the cysts remained viable and, at the convenience of the investigator, could be used in experiments during the following week. Individual hanging-drop cultures were prepared by pipetting a

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drop of cyst suspension, about 20 mm.^{3} in volume, onto the underside of a coverslip; the latter was then sealed to a depression slide with melted paraffin.

In experiments where numerous cultures were required, the procedure was altered, as follows. Glass plates, 5″ × 5″ × 0.06″, were covered with thin films of petrolatum and sterilized. "Sitting-drops" of about 20 mm.^{3} volume were pipetted in rows on each plate. A brass ring, 4″ in diameter × 0.125″ wide × 0.08″ thick, was placed on the glass plate surrounding the drops, and another 5″ × 5″ × 0.06″ glass plate set on top of the ring. The chamber, thus formed, was sealed with

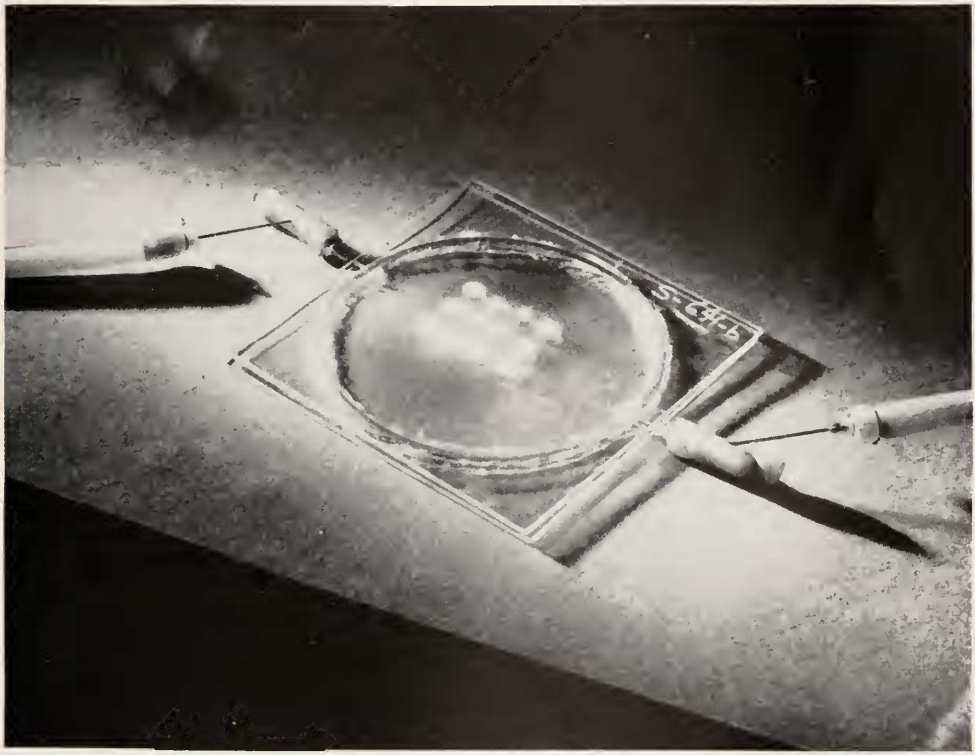

FIGURE 1. Culture chamber for studying the effects of various gases on sitting-drop preparations.

melted paraffin. In experiments in which cysts were exposed to specific gaseous environments, the culture chambers were modified by the addition of inlet and outlet tubes to permit the circulation of suitable gas mixtures (Fig. 1).

2. Quantitative determination of the rate of spermatogenesis in a population of cysts [3]

When suspended in blood containing the growth and differentiation hormone ("active blood"), the spermatocytes undergo meiosis and spermiogenesis during

[3] We gratefully acknowledge the assistance of Mr. Ned Feder in the development of this quantitative method.

the course of about two weeks. Most of the cysts contain fully differentiated spermatozoa by the end of this period. In order to quantitate the rate of spermatogenesis, the stage of development of each cyst in the hanging- or sitting-drops was recorded at frequent intervals during the period of culture.

Four successive stages in the development are easily recognized (see Fig. 1, Schmidt and Williams, 1953). Stage I includes primary and secondary *spermatocytal* cysts. Stage II includes spherical *spermatidal* cysts that have not yet begun to elongate. Stage III includes partially elongated *spermatidal* cysts less than twice as long as wide. Stage IV includes elongated *spermatidal* cysts more than twice as long as wide.

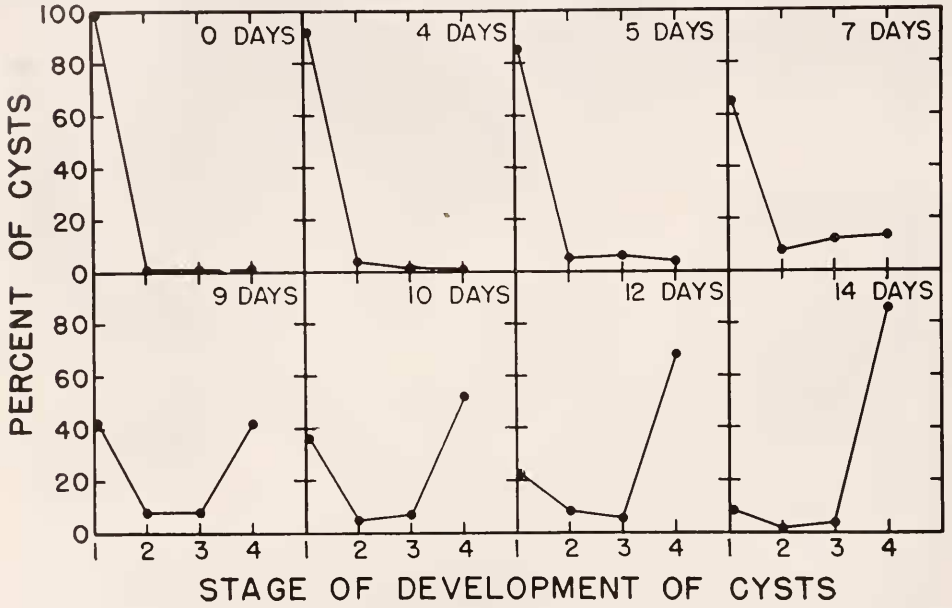

FIGURE 2. Day-to-day profiles during the normal development of a population of 600 cysts. For explanation see text.

As soon as a culture was prepared, the cysts were counted and identified under the low power objective of the compound microscope. This procedure was repeated at one- to three-day intervals. The results, when plotted, produced a series of "profiles" which described the composition of a particular population of cysts as a function of time.

The procedure is illustrated in Figure 2, which records the day-to-day profiles during the normal development of a population of approximately 600 cysts distributed in three hanging-drops. At zero days there was no development. But after five days, 19 per cent of the cysts had undergone meiosis and, of these, about 5 per cent had progressed to stage IV. Thereafter, the percentage of cysts in stage I continued to drop rapidly while that in stage IV increased. After nine days, the two stages were present in nearly equal numbers; after fourteen days, 86 per cent of the cysts had progressed to stage IV.

The determination of the relative rates of spermatogenesis (including both meiosis and spermiogenesis) under various experimental conditions was ordinarily carried out when 30 to 40 per cent of the cysts in a parallel control population had reached stages III or IV. The per cent of cysts in stages III or IV in the experimental population was compared with the corresponding determination on the control population. The relative rate of spermatogenesis was calculated as per cent of that observed in the control.

3. Reagents

Stock solutions were prepared in distilled water and the pH adjusted to 6.8–7.2 by the addition of NaOH or HCl. All heat-stable reagents were autoclaved before use. Heat-labile reagents were prepared in sterile distilled water and neutralized with sterile solutions of acid or base.

4. Gases

Compressed gases were obtained in commercial cylinders and assayed as follows:

Nitrogen (Airco), 99.5% nitrogen plus less than 0.5% oxygen. Nitrogen (Linde), 99.99% nitrogen plus less than 0.01% oxygen. Oxygen (Airco), 99.5% oxygen plus less than 0.5% nitrogen. Carbon monoxide (Matheson Co.), 96.8% carbon monoxide, 0.36% carbon dioxide, 0.97% hydrogen, 1% nitrogen, 0.8% saturated hydrocarbons, 1.19 mg. iron/liter, 0.32 mg. sulfur/liter. Prior to its use, the carbon monoxide was filtered through 10% sodium hydroxide solution to remove the carbon dioxide and the iron carbonyl compounds.

Mixtures of oxygen, nitrogen, and carbon monoxide were prepared by compression in steel cylinders.

5. Use of inhibitors

A fresh suspension of cysts was prepared in active blood and divided into 0.09-ml. aliquots in small test tubes. To each tube was then added 0.01 ml. of an appropriate concentration of an inhibitor. The resulting 0.1-ml. mixture was drawn into a pipette and distributed in five drops of about 20 $mm.^3$ each on a sterile $5'' \times 5''$ glass plate, as described in section 1. The preparations were then maintained at 25° C. in an incubator, and profile counts performed at intervals of one to three days. Three drops were selected containing a total of 300 to 600 cysts. The stage of development of each cyst was scored. Cysts and blood from the same series of donors were used in the preparation of parallel control cultures to which nothing save phenylthiourea was added. In the experimental results, the concentrations of the various inhibitors are recorded as the initial concentrations in the cultures. No attempt was made to define their distribution; *i.e.*, binding to cells, volatilization, or possible loss through degradation.

6. Addition of gases

About ten drops of a fresh cyst suspension were enclosed in the chamber illustrated in Figure 1. The glass plates were sealed with petrolatum and held together with clamps (not shown in the figure). Fifty to seventy-five volumes of

an appropriate gas mixture saturated with water vapor were flushed through the chamber; the latter was then submerged in a water bath at 25° C. In experiments utilizing extremely low oxygen tensions, the chambers were flushed out daily to prevent the respiration of the cells from diminishing the oxygen tension significantly.

7. Photo-reversibility of carbon monoxide inhibition

In studies of the photo-reversibility of the carbon monoxide inhibition of spermatogenesis, a 150-watt incandescent lamp was utilized. The light was gathered by a reflector and focussed by means of a biconvex lens; it was then passed through a No. 3962 Corning filter and several centimeters of water to cut off the infrared. Each chamber was submerged in a 25° C. water bath and illuminated from above by a separate lamp. Control chambers were maintained in darkness by an envelope of aluminum foil.

Results

Preliminary experiments were performed with each agent to define the range of concentration which inhibited spermatogenesis. Concentrations greater than 10^{-2} *M* were not investigated unless a specific activity at higher concentrations had previously been reported. A total of approximately 200 experiments was performed; each concentration of each agent was tested in duplicate or triplicate.

Table I

The effects of temperature on in vitro spermatogenesis

Temperature	Time required for initiation of development
10° C.	No development after 31 days
15° C.	23 days
20° C.	7 days
25° C.	3½ days
30° C.	3½ days

1. Effects of temperature

The results of the temperature studies are presented in Table I. When cysts were cultured at 10° C., no development was evident after 31 days. At 20° C. the first signs of development were detectable after 7 days, while at 25° C. and 30° C., development began in 3½ days. It is also of interest that after culture at 10° C. for as long as 15 days, the cysts resumed the normal tempo of development when returned to 25° C.

2. Effects of metabolic inhibitors

Figure 3 illustrates the results of a typical experiment in which suspensions of cysts were exposed for seven days to graded concentrations of an inhibitor—in this particular case, 2, 4-dinitrophenol. Development in the presence of 10^{-7} *M* proceeded at the same rate as that of controls; in 10^{-6} *M* there was definite inhibition; in 10^{-5} *M* only a few cysts underwent differentiation; in 10^{-4} *M* the cells were killed.

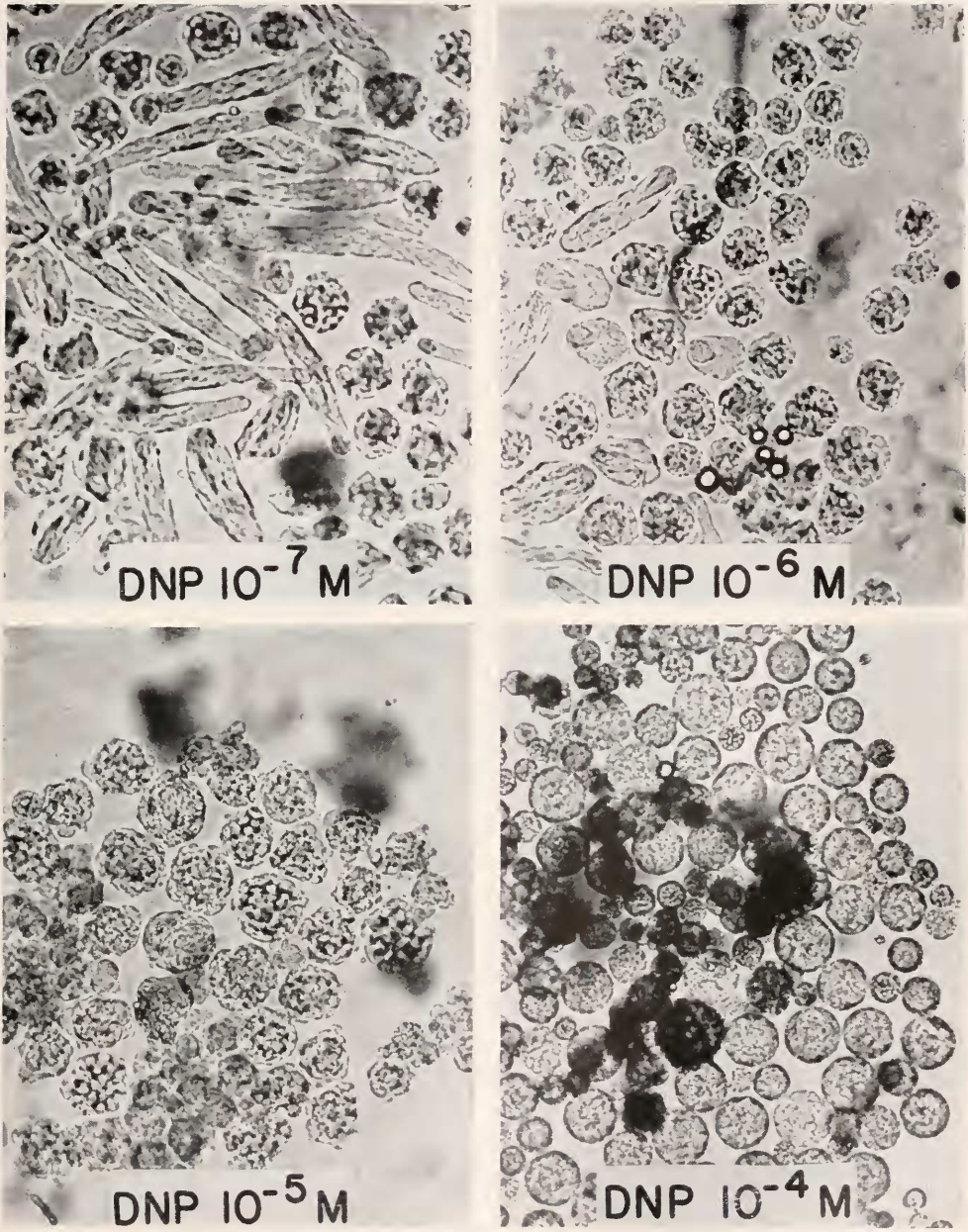

FIGURE 3. Fresh unstained cysts after seven days of culture in graded concentrations of 2, 4-dinitrophenol. × 65.

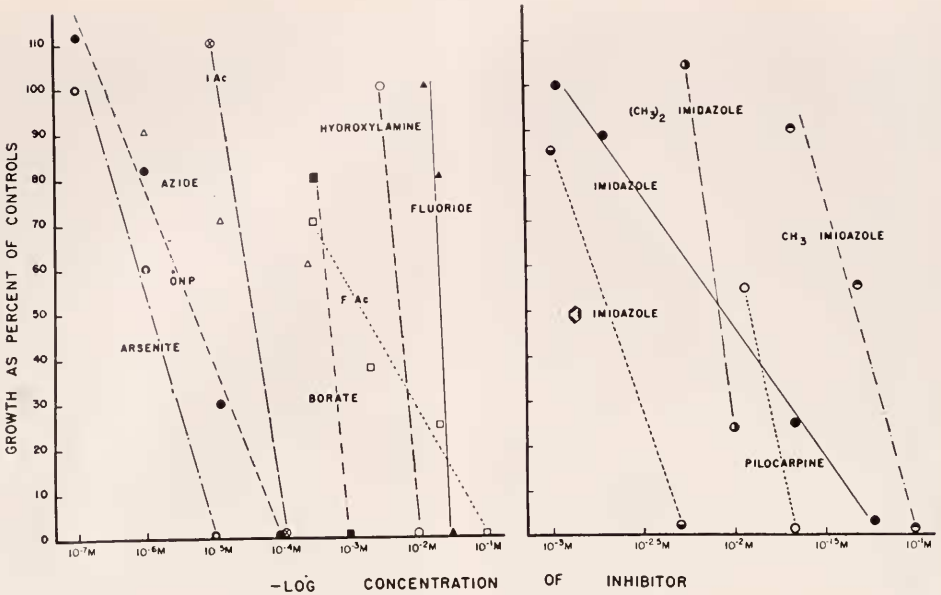

FIGURE 4. Effects of metabolic inhibitors on *in vitro* spermatogenesis.

TABLE II

The effects of twenty metabolic inhibitors on in vitro spermatogenesis (pH 6.8 to 7.2)

Inhibitor		Approximate concentration required for 50% inhibition of spermatogenesis ($\times 10^{-5}$ *M*)
1. Sodium arsenite		0.2
2. 2,4-dinitrophenol		0.7
3. Sodium iodoacetate		4
4. Sodium tetraborate		30
5. Sodium azide		80
6. Sodium fluoroacetate		80
7. Benzimidazole		200
8. Hydroxylamine hydrochloride		300
9. Semicarbazide hydrochloride		500
10. Imidazole		700
11. Dimethyl imidazole		700
12. Pilocarpine		1000
13. Sodium fluoride		3000
14. Methyl imidazole		5000
15. Ferrideuteroporphyrin		0.02 mg./ml.
16. Sodium pyrophosphate	No inhibition at	100
17. Coumarin	Slight inhibition at	500
18. Phenylthiourea*	No inhibition at	1000
19. Sodium malonate and malonamide	No inhibition at	5000

* All cultures were saturated with this drug for the purpose of blocking tyrosinase.

The quantitative results of a large number of similar experiments with different inhibitors are summarized in Figure 4. Development is recorded as per cent of that observed in control cultures as a function of the logarithm of the concentration of inhibitor. Although the range of effective concentrations differed considerably in the case of the several inhibitors, for each agent there appeared to be a roughly linear decrease in the rate of spermatogenesis as a function of the logarithm of inhibitor concentration. Table II tabulates the approximate concentrations of 20 chemicals that inhibited the rate of spermatogenesis by 50 per cent.

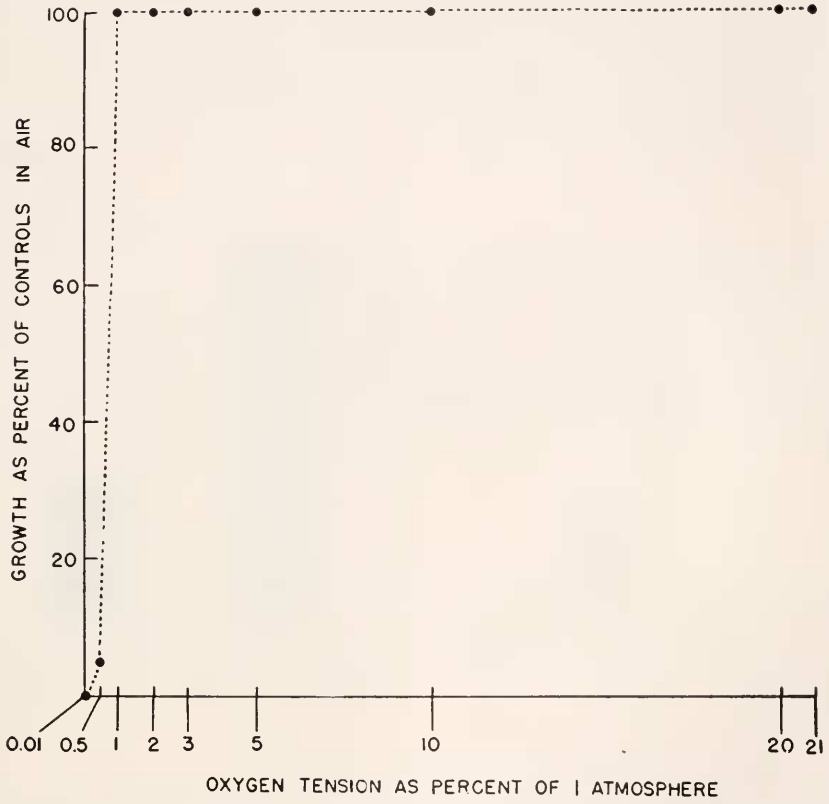

FIGURE 5. Diagrammatic summary of the effects of oxygen tension on *in vitro* spermatogenesis.

3. *Effects of oxygen tension*

Experiments testing the effects of oxygen tension on *in vitro* spermatogenesis are diagrammatically summarized in Figure 5. Oxygen at tensions between 21 per cent of an atmosphere (the normal tension in air) and 1 per cent of an atmosphere permitted spermatogenesis to proceed at the normal rate. As the oxygen tension was decreased below 1 per cent, considerable inhibition was observed. In 0.5 per cent oxygen the rate of development was only about 5 per cent of normal; in 0.01 per cent oxygen, development was completely inhibited.

4. Effects of carbon monoxide

Figure 6 records the effects of carbon monoxide on spermatogenesis. It will be observed that the inhibition depended not only on the pressure of carbon monoxide, but also on the pressure of oxygen that was simultaneously present. Thus, while a mixture of 90 per cent carbon monoxide plus 10 per cent oxygen (9:1) retarded the rate of development by 70 per cent, 90 per cent carbon monoxide plus 5 per cent oxygen (18:1) caused a 98 per cent inhibition.

In another series of experiments, cysts were exposed to 90 per cent carbon monoxide plus 10 per cent oxygen for five days and then returned to air. In many cysts the inhibition was immediately reversed.

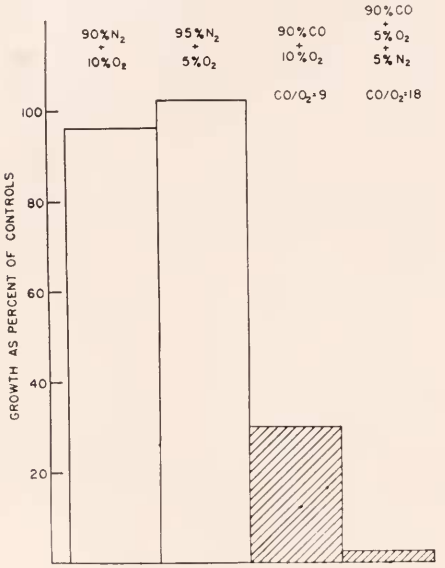

FIG. 6. Effects of exposure to various carbon monoxide/oxygen ratios on *in vitro* spermatogenesis in the dark.

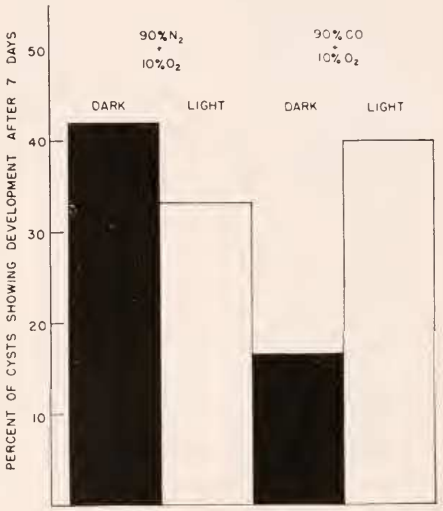

FIG. 7. Effects of light on the carbon monoxide inhibition of *in vitro* spermatogenesis.

5. Photo-reversibility of the effects of carbon monoxide

Because of their transparency the cysts afforded optimal material for testing the light reversibility of the carbon monoxide inhibition. Four chambers were used containing, respectively, 90 per cent nitrogen in the light, 90 per cent nitrogen in the dark, 90 per cent carbon monoxide in the light, and 90 per cent carbon monoxide in the dark. Ten per cent oxygen was present in all chambers. The results, recorded in Figure 7, show that light completely reversed the inhibitory effects of carbon monoxide.

DISCUSSION

The experimental results are relevant to considerations of the intermediary metabolism of both meiosis and spermiogenesis and to previous findings on intact insects.

1. Temperature

In vitro spermatogenesis showed substantially the same response to temperature as was encountered in the adult development of the animal as a whole. Both proceed most rapidly at environmental temperatures of 25° to 30° C. (Williams, unpublished observations) and are reversibly inhibited by low temperatures.

2. Glycolytic system

Though the anti-glycolytic agents, fluoride and iodoacetate, are not wholly specific, their potent inhibition of spermatogenesis suggests that the usual hexose-triose pathway of carbohydrate metabolism operates in the sex cells. However, it is clear that spermatogenesis is an aerobic process; the glycolytic reactions are therefore presumably concerned with the mobilization of substrates for oxidative breakdown.

3. Tricarboxylic acid cycle

The inhibition by arsenite, fluoroacetate, hydroxylamine, and iodoacetate is good evidence that the tricarboxylic acid cycle is utilized in spermatogenesis. In intact Cecropia pupae and developing adults, several components in this cycle have previously been demonstrated; namely, succinic dehydrogenase, malic dehydrogenase, and fumarase (unpublished observations) and coenzyme I (Jencks and Williams, unpublished observations). Moreover, fluoroacetate is known to be extremely poisonous for intact Cecropia at all stages in the life history (Williams, unpublished observations).

4. Phosphate bond energy

The inhibition by low concentrations of 2,4-dinitrophenol strongly suggests that spermatogenesis is energized by phosphate bond energy. This conclusion is consistent with the high toxicity of 2,4-dinitrophenol for intact Cecropia at all stages of development (Williams, unpublished observations).

We likewise suspect that the toxicity of azide is attributable, at least in part, to its inhibition of phosphate fixation (Loomis and Lipmann, 1949). Though azide is also known to inhibit the cytochrome system, the drug is extremely toxic when injected into diapausing Cecropia pupae (Williams, unpublished observations), where the function of the cytochrome system is not prerequisite to survival (Schneiderman and Williams, 1952).

5. Terminal oxidases

a. The experiments testing the effects of oxygen tension demonstrate that spermatogenesis is an aerobic process which apparently cannot be energized by glycolytic reactions. The short distances for the diffusion of oxygen in the *in vitro* preparations enabled the gas to saturate the terminal oxidase when the ambient gas phase contained oxygen at a tension of only 8 mm. of Hg. This finding contrasts sharply with that observed on the intact insect where oxygen is required to diffuse considerable distances through the tracheal system. Consequently, the

adult development of Cecropia is inhibited when the ambient oxygen tension falls below 38 mm. Hg (5 per cent of an atmosphere) (unpublished observations). The fact that spermatogenesis proceeds at finite, though extremely low, tensions of oxygen is presumptive evidence that the terminal oxidase is cytochrome oxidase—an enzyme which, unlike other oxidases, is saturated at oxygen tensions between 0.25 and 2.5 mm. Hg (Winzler, 1941).

b. The failure of phenylthiourea, a powerful inhibitor of tyrosinase, to block spermatogenesis is in agreement with observations on intact Cecropia. Here phenylthiourea inhibits neither adult development (Williams, unpublished observations) nor respiration (Sussman, 1952). It seems safe to conclude that tyrosinase is not a terminal oxidase in processes responsible for the development of Cecropia *in vitro* or *in vivo*.

c. According to Pappenheimer and Williams (1952), certain imidazoles such as pilocarpine exert their effects by complexing ferriprotophyrin, thereby making this prosthetic group unavailable for the synthesis of heme-containing enzymes such as the cytochromes. The inhibitory action of these imidazoles on spermatogenesis (Fig. 4) roughly parallels their effects in blocking the development of the intact insect.

d. The inhibition of spermatogenesis by ferrideuteroporphyrin is probably attributable to its competitive inhibition of ferriprotoporphyrin (Pappenheimer and Williams, 1952). This inference once again points to the role of enzymes containing ferriprotoporphyrin in the metabolism of spermatogenesis; *e.g.*, certain components of the cytochrome system such as cytochrome *b*. The effects observed *in vitro* are analogous to those observed in intact Cecropia where ferrideuteroporphyrin inhibits adult development and other processes requiring cytochrome-mediated respiration.

e. Carbon monoxide's inhibition of spermatogenesis directly parallels its action on intact Cecropia during the period of adult development (Schneiderman and Williams, 1952). Since *in vitro* spermatogenesis was resumed upon return to air, it is clear that carbon monoxide blocked or retarded development without killing. The carbon monoxide inhibition of spermatogenesis was dependent on the carbon monoxide/oxygen ratio. The same finding has been demonstrated for the inhibition of adult development in the intact insect. Finally, the carbon monoxide inhibition of *in vitro* spermatogenesis was reversed by light—an effect which we have duplicated in the intact insect and shall describe in a subsequent communication.

Only one substance is affected by carbon monoxide in this manner; namely, cytochrome oxidase. We therefore conclude that cytochrome oxidase is the terminal oxidase in the morphogenesis of the testicular tissue, as in the insect as a whole.

The photograph in Figure 1 was made in collaboration with Dr. Roman Vishniac and is used with the permission of *Time*, Inc.

Summary

1. The effects of temperature, metabolic inhibitors, oxygen tension, and carbon monoxide were studied in relation to the *in vitro* spermatogenesis (meiosis and spermiogenesis) of the male sex cells of the Cecropia silkworm.

2. A method is described for the quantitative study of the rate of spermatogenesis.

3. The optimum temperature for spermatogenesis is 25° to 30° C.

4. An appraisal of the effects of twenty diverse metabolic inhibitors revealed that the glycolytic system mobilizes substrates for spermatogenesis, that the tricarboxylic acid cycle is apparently the main pathway of acetate utilization, and that oxidative phosphorylation energizes spermatogenesis.

5. Spermatogenesis is shown to be an aerobic process, the terminal oxidase mediating the respiration being saturated by oxygen at tensions lower than 8 mm. of Hg.

6. Spermatogenesis is blocked or retarded by several inhibitors of the cytochrome system; the inhibition by carbon monoxide is light-reversible.

7. The findings on the isolated sex cells are compared to the effects of the same series of agents on the intact insect. The biochemical systems responsible for the meiosis and differentiation of spermatocytes *in vitro* are apparently the same as those which serve the development of the insect as a whole.

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